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TITLE: Development and Commercialization of Analyte Specific Reagents (ASRs)for the Diagnosis of Selected Arthropod-Borne Viruses on FDA-Cleared Real-time PCR Platforms

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14. ABSTRACT

The primary goal of the Phase II Contract was to continue to design, manufacture, and test real time polymerase chain reaction (qPCR) reagents for detection of one or more of six potentially deadly arbovirus pathogens: Dengue, Rift Valley Fever, Chikungunya, Crimean-Congo Hemorrhagic Fever, Sand Fly Fever, and Tick-Borne Encephalitis viruses. The qPCR reagents being provided in Phase II are additional primers and probes ("signature sets") sets for qPCR applications, as well as positive controls (synthetic targets) for the assays. Work product in Phase II included: (1) design and synthesis of a synthetic target representing all four Dengue serotypes; (2) cloning of a plasmid control for the new RT-qPCR signature set; (4) qPCR testing of the new Dengue signature set, synthetic template, and plasmid control; (5) determination of efficiency, Linearity of Range (LOR), and Limit of Detection (LOD) for all eight qPCR signature sets; (6) bioinformatic analysis and alignment of sequences published for the high priority agents listed; (7) design and synthesis of a new "consensus" qPCR signature set for Dengue types 1-4 ("DENcon") based on bioinformatics analysis of 42 Dengue subtypes; (8) design and synthesis of a synthetic template for the "consensus" Dengue signature set; (9) design and synthesis of a control plasmid ("ARBO9") containing sites for all eight signature sets; (10) the testing of RNA generated from ARBO9 as a synthetic target; and (11) stability testing of all reagents, including positive controls.

15. SUBJECT TERMS
Arbovirus detection; qPCR; RT-qPCR

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Research Objectives

The objective of this Grant is to develop and test one step reverse transcriptase real time polymerase chain reaction (RT-qPCR) assays for six arbovirus pathogens, and to manufacture and supply the successful assays to the Army as Analyte Specific Reagents (ASRs). The designated pathogens are Dengue fever (DEN), Rift Valley Fever (RVF), Chikungunya (CHIK), Crimean-Congo Hemorrhagic Fever (CCHF), Sand Fly Fever-Toscana (SFF), and Tick-Borne Encephalitis-Central European (TBE-CE).

Description of the Problem

While stationed outside of the United States, military forces are exposed to infectious disease agents not present at home, and for which the individuals have little or no immunity. Contracting such diseases can cause moderate to severe illness, even death, thus becoming a threat to U.S. military operations around the world. Rapid and specific identification of the infectious agent can be critical, as both treatment of the infected individual and the preventative measures taken to guard against spreading the infection vary depending on the pathogen in question. Diagnosis of the disease and identification of the causative pathogen is particularly difficult in foreign field settings, where the appropriate reagents, instrumentation, power, storage, and environmental control (refrigeration, etc) needed for most diagnostic reagents are difficult to obtain. Moreover, assays for many of the pathogens do not currently exist, as there is little economic incentive for a U.S. company to develop approved diagnostic tests for rare diseases in other countries.

Research Goals

The overall goals include:

1. design and synthesis of multiple qPCR probes and primers for each of the six pathogens;
2. design and synthesis of positive controls (DNA and RNA) for each pathogen signature;
3. optimization of primers/probe signatures against positive controls using qPCR to allow all assays to be run under identical conditions, consistent with the existing JBAID platform;
4. formatting of reagents as lyophilized field-stable Analyte Specific Reagents;
5. stability testing of the reagents under mock field conditions; and
6. manufacture of packaged test lots for the military.

Expected Impact

If RT-qPCR assays are successful for these pathogens, diagnosis of the diseases will be shortened from days or weeks to 2-3 hours. This allows the infected military personnel to be treated and quarantined quickly, reducing both the severity and the spread of the disease. The assays can also be used to analyze environmental samples (mosquitoes, ticks, water, etc) to pinpoint the source of the pathogen, allowing preventative treatments.

Technical Approach

Based on both literature reports and genomic alignment of serotypes, primers and dual labeled probes were designed, synthesized and tested. Positive controls were made in plasmids for initial testing. The plasmids were also used to make RNA transcripts of the controls, allowing the assay to be run as both qPCR and RT-qPCR. Performance of the eight signature sets against plasmid (DNA) target are summarized in Table 1; performance of one of the signature sets against RNA transcripts compared to plasmid is summarized in Table 2. Synthetic templates (linear dsDNA 80-100 bases in length) were also made and tested. Stability studies through 7 months dry at ambient temperature indicated the probes and primers were stable.

Table1 : Performance of the Eight Signatures Sets against ARBO9 Plasmid Target

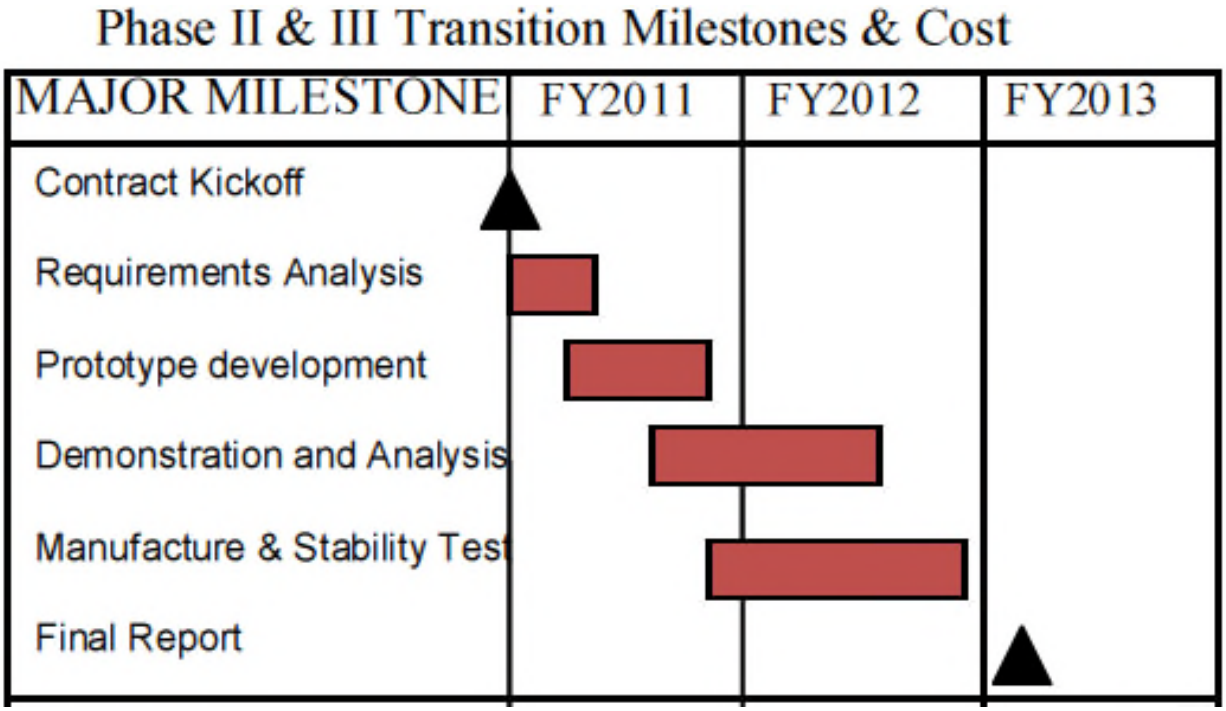
Signature	LOD	ROL (R^2)	Cycle Efficiency
DENcon	<10 copies	0.999	95%
DEN Wu	<10 copies	0.999	98%
DEN2	<10 copies	0.999	104%
CCHF5	<10 copies	0.998	107%
Chik	1 copy	0.998	107%
RVF	1 copy	0.999	103%
TBE	<10 copies	0.999	100%
SFF	1 copy	0.999	100%

Table 2: Initial Performance against ARBO9 RNA Transcript using RT-qPCR

Signature	Position in RNA*	C_q (10^8 copies positive)	LOD (lowest copy #)	ROL (R^2)	Cycle Efficiency
CCHF (5'-end)	6	9 cycles	10	0.999	102%
DEN2	73	13 cycles	1000	0.999	95%
DENcon	146	8 cycles	100	0.997	96%
CHIK	220	9 cycles	10	0.999	105%
RVF	301	8 cycles	1	0.999	99%
TBE	406	9 cycles	10	0.999	91%
SFF	497	8 cycles	1	0.998	102%
DENwu	596	9 cycles	1	0.997	99%

*nucleotides from 5'-end of the RNA to the 5'-start of the target

Schedule Graphic



Deliverables

Signature sets were manufactured under GMP as Analyte Specific Reagents. For each of the six pathogens, 100 tubes (enough for 20,000 assays) were sent to Dr John Lee, the COR, for his testing.